

Exposure of Arabidopsis plants to dioxin results in a *Wrinkled* seed phenotype that is likely due to 20S proteasomal degradation of WRI1

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Highlight

Plants are increasingly exposed to the organic persistent pollutants, the dioxins. Here we show that the exposure of *Arabidopsis thaliana* to such pollutants resulted in specific perturbations of seed development.

Abstract

We have investigated the effects of dioxins on seed development in *Arabidopsis thaliana*. Dioxins are highly toxic persistent organic pollutants bioaccumulated by both plants and animals that also cause severe developmental abnormalities in humans. Plants were exposed to various concentrations of the most toxic congener of dioxins, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and the effects on seed development were analysed in depth at transcriptome, proteome and metabolome levels. Exposure to dioxin led to generalised effects on vegetative tissues plus a specific set of perturbations to seed development. Mature seeds from TCDD-treated plants had a characteristic 'Wrinkled' phenotype, due to a two-thirds reduction in storage oil content. Transcriptional analysis of a panel of genes related to lipid and carbohydrate metabolism was consistent with the observed biochemical phenotypes. There were increases in *WRI1* and *LEC1* expression but decreases in *ABI3* and *FUS3* expression, which is puzzling in view of the low seed oil phenotype. This anomaly was explained by increased expression of 20S proteasome components that resulted in a substantial degradation of *WRI1* protein, despite the upregulation of the *WRI1* gene. Our findings reveal novel effects of dioxins that lead to altered gene regulation patterns that profoundly affect seed development in *Arabidopsis*.

Keywords

Arabidopsis, Dioxin, Phytotoxicity, Proteasome, Seed metabolome, *Wrinkled*, *WRI1*.

Introduction

Dioxins are hydrophobic compounds that tend to bioaccumulate in fatty tissues of animals after ingestion or following direct uptake from the environment. In terrestrial and aquatic environments subject to dioxin pollution, elevated levels of dioxins can be found in marine organisms harvested as seafood and in herbivorous livestock, such as cattle, that are sources of meat and dairy products. From these sources dioxins can readily enter human food chains and thereby constitute a potentially serious health risk. Indeed, dioxins are well documented as causing irreversible biological damage in humans and other animals and can also have significant wider ecological, environmental and economic impacts (Desforges *et al.*, 2016; Glazer *et al.*, 2016).

In animals, it has been shown that dioxins specifically interact with a lipid-soluble ligand-dependent ubiquitin ligase complex that includes a dioxin receptor termed AhR (Ohtake *et al.*, 2007). The AhR ligands, such as dioxins, can directly modulate steroid hormone signaling pathways and also affect specific transcriptional regulatory networks (Ohtake *et al.*, 2007). Plants do not have AhR genes and, although they are affected by dioxin exposure, there have been very few studies on any wider effects on plant development. Our previous work on plant-dioxin interactions described above (Hanano *et al.*, 2016a) was done with date palm, which is a large, slow growing perennial tree species that is not amenable to molecular developmental studies. We therefore selected the model plant *Arabidopsis thaliana* for more detailed analysis of dioxin exposure at the biochemical and genetic levels.

Plants can be exposed to environmental dioxins and can accumulate them in their root system with more hydrophobic congeners being taken up to a greater extent (Inui *et al.*, 2011; Zhu *et al.*, 2012). *Arabidopsis* plants can also absorb such xenobiotics from the external environment, and tend to accumulate them in leaves, seeds and roots (Hanano *et al.*, 2015a). At the subcellular level, one obvious way in which dioxins might interact with plants is via their hydrophobic components, the most notable of which are the cytosolic lipid droplets (LDs) that are ubiquitous in most living organisms (Murphy,

2012). The major site of LD accumulation in plants is in seed tissue where these organelles can represent 50-60% of the total dry weight in some mature seeds.

We have recently demonstrated that LDs extracted from date palm seeds can act as extremely effective sequestration agents for the dioxin, TCDD (Hanano *et al.*, 2016a). We also found that exposure of date palm seedlings to TCDD resulted in a strong transcriptional induction of some members of the caleosin gene family. Caleosins are multifunctional lipid-, haem- and calcium-binding proteins that are major components of the LD proteome in plants, where they are inserted into the phospholipid monolayer that surrounds the triacylglycerol (TAG) core of the LDs (Murphy, 1993). Caleosins are also found on the bilayer membrane within the cell and can have peroxygenase activities (Hanano *et al.*, 2006). Caleosin genes are strongly upregulated during the TAG accumulation phase of seed development. However, they are also highly responsive to a range of biotic and abiotic stresses and are involved in physiological processes such as stomatal control, transpiration, seed germination and G protein signalling (Poxleitner *et al.*, 2006; Aubert *et al.*, 2010; Ehdaei *et al.*, 2014). It is possible that caleosins are directly involved in the plant response to dioxins at several different levels. For example, caleosins might help stimulate LD accumulation in order to assist toxin sequestration (Hanano *et al.*, 2016b). Alternatively, or perhaps additionally, caleosins may act as part of an oxylipin signalling pathway that is involved in the overall stress response to the toxin (Bagchi and Stohs, 1993).

The vegetative growth phenotype resulting from TCDD exposure was investigated in a previous report (Hanano *et al.*, 2015b), which showed that administration of TCDD to *Arabidopsis* plants caused reductions in fresh weight and chlorophyll content plus enhanced hydrogen peroxide production and a massive stimulation of leaf anti-oxidant enzyme activities. The TCDD mainly accumulated in rosette leaves and mature seeds with much less found in stems, flowers and immature siliques. The roots of TCDD-exposed plants showed increased lateral growth (Zhu *et al.*, 2012) but there was a delay in flowering and reduced seeds yield, oil content and overall seed vitality (Hanano *et al.*,

2015b). These initial studies showed that, as in animals, dioxin exposure was associated with a complex suite of symptoms in plants, many of which may be related to oxidative stress. However, we also suspected that, as in animals, dioxins might also have more specific effects related to reproduction. For example, we noted that dioxin exposure strongly and reproducibly affected seed development and storage product accumulation, and particularly on the amount of storage oil in mature seeds. The accumulation of oil in the seeds of plants such as *Arabidopsis* is regulated by a group of transcription factors that are responsible for directing the flux of assimilates imported into developing seeds towards TAG rather than other potential storage products such as starch or proteins (Maeo *et al.*, 2009; Ma *et al.*, 2013). For example, *WRINKLED1* (*WRI1*) genes are downstream members of a group of transcription factors that exert considerable control over the latter part of the glycolytic pathway as well as over carbon flux towards fatty acid and TAG biosynthesis. *WRI1* may also be involved in the formation of the LD proteins, such as oleosin and caleosin, which mediate the assembly of TAG into stable lipid droplets (Santos-Mendoza *et al.*, 2008; Baud and Lepiniec, 2010). The upstream transcription factor *LEAFYCOTYLEDON2* (*LEC2*) is proposed to regulate *WRI1*, but also has a role in regulating TAG biosynthesis genes. In turn, *LEC2* and *WRI1* are the targets of master regulator genes such as *LEC1*, *PKL* and *B3* domain genes (Swaminathan *et al.*, 2008; Peng and Weselake, 2013).

In this study we examined the effects of the dioxin, TCDD, on mature *Arabidopsis* plants and characterized several changes in patterns of seed development at the biochemical and transcriptional levels. Interestingly, these changes included alterations in expression of several transcription factor genes involved in mediating the flux of assimilates towards storage compounds. In the case of the transcription factor *WRI1*, TCDD-mediated increased transcription of its gene was counterbalanced by an upregulation of the expression of 20S proteasome genes, which led to rapid degradation of the newly synthesised *WRI1* protein following upregulation of the classic ubiquitin-proteasome

system. This has interesting analogies with the reported effects of dioxins on protein degradation via the ubiquitin ligase complex in animal systems (Ohtake *et al.*, 2007).

Materials and Methods

Plant material, culture conditions, TCDD-treatment and microscopy

Arabidopsis thaliana ecotype Columbia 0 (Col-0) seeds were firstly sterilised with 70% alcohol and sown in pots containing a sterilized mixture of potting soil, vermiculite and perlite (50:30:20 v/v/v). Seeds were induced to germinate by incubating pots at 4 °C for two days and transferring to growth chambers. Plants were grown at 20/15 °C day/night temperatures under a 16h/8h light/dark regime (100 mmol m⁻² s⁻¹). 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD dissolved in toluene at 10 µg mL⁻¹, purity 99%) was purchased from Supelco Inc., USA. Plants were irrigated with TCDD solutions (0, 10 and 50 ng L⁻¹) twice a week. These concentrations were chosen as they are below the 'levels of action' for dioxins in soil as determined by the US Environmental Protection Agency that range between 500 and 1,000 ppt (ng L⁻¹). Responses to TCDD were analysed during seed development stages starting from 5 days to 17 days post flowering (at two-day intervals) according to Focks and Benning (Focks and Benning, 1998). For each treatment or control, about 10 seeds were sown in individual pots containing a sterilized mixture of potting soil, vermiculite and perlite (50:30:20 v/v/v). Fifteen pots were prepared and about 150 plants produced from each treatment (10 and 50 ng L⁻¹) and for controls. Plants were grown at 20/15 °C day/night temperatures under the same conditions as mentioned above. Under these conditions, control plants began flowering on week 4 while plants treated with 10 or 50 ng L⁻¹ of TCDD began flowering on week 5 and 6, respectively. To harvest siliques of defined developmental stages, individual flowers were marked by coloured threads on the day of flowering. About 10 grams of siliques were collected at each development stage from treated and control plants. Representative seeds were carefully separated from siliques and valves, replaced in Eppendorf tubes and frozen in liquid nitrogen and kept at – 80 °C for further analysis. For each time point, three individual extractions were done and measurements

carried out in triplicate. Microscopic imaging for seeds was performed at 30× magnification using a LEICA MPS60 microscope and with a Scanning Electron Microscope (SEM) using a Vega II XMU, TESCAN, Czech Republic at magnifications of 200× and 500×.

Total lipid, proteins and carbohydrate analysis

Lipids, proteins and carbohydrate were extracted and analysed as described by Focks and Benning (Focks and Benning, 1998). For TLC-analysis of triacylglycerol, 50 seeds were ground in liquid nitrogen and lipids were extracted in 200 µL of chloroform/methanol/formic acid (10/10/1, v/v/v). Following the extraction with 50 µL of 1 M KCl and 0.2 M H₃PO₄ and separation of the organic and aqueous phases by centrifugation at 16,000 × *g* for 5 min, the lipids in the lower phase were separated on a silica TLC plate (TLC Al foils, Sigma-Aldrich, Germany) developed with hexane/diethylether/acetic acid (60/40/1, v/v/v). Lipids were visualized by staining with iodine vapour and compared with a commercial standard TAG mixture (Sigma-Aldrich, Germany). Proteins were extracted according to Focks and Benning (Focks and Benning, 1998). Total protein was quantified in 200 µL supernatant using the Bradford assay (Bio-Rad) (Bradford, 1976). For starch extraction, the same amount of seeds were ground in liquid nitrogen and homogenized in 500 µL of 80% (v/v) ethanol then incubated at 70 °C for 90 min. The homogenate was centrifuged at 16,000 × *g* for 5 min and the resulting supernatant, representing the soluble sugars fraction, was transferred to a new test tube. The solvent of the combined supernatants was evaporated at room temperature under a current of nitrogen. The residue was dissolved in 50 µL of water. The pellet containing starch, was homogenized in 200 mL of 0.2 N KOH, and the suspension was incubated at 95 °C for 1 h to dissolve the starch. The quantification of soluble sugars and starch was done as described before (Focks and Benning, 1998).

Fatty acid analysis

Fatty acids were quantified by a GC-MS system (Agilent 6850) of the corresponding fatty acyl methyl esters. For that, 50 seeds were ground in liquid nitrogen then transferred into a glass reaction tube and incubated in 1 mL of 1 N methanolic HCl at 80 °C for 2 h. Fatty acyl methyl esters were extracted into 1 mL of hexane following the addition of 1 mL of 0.9% (w/v) NaCl. Myristic acid was used as an internal standard. The resulting fatty acid methyl esters (FAMES) were extracted in hexane and analysed by a GC-MS (Agilent 6850) as described previously (Murayama *et al.*, 2006). Fatty acids were identified and their relative amounts were calculated from their respective chromatographic peak areas compared with a standard FAME mixture.

Preparation of plant enzymes extracts and enzymatic assays

Plant extracts from approximately one gram of siliques taken on 5 to 17 days after flowering were prepared according to (Focks and Benning, 1998). The following enzymes were assayed as previously described: hexokinase according to the method of Renz *et al.* (Renz *et al.*, 1993); glucose-6-phosphate dehydrogenase according to the method of Burrell *et al.*, (Burrell *et al.*, 1994); AGP-Glc-pyrophosphorylase according to the method of Zrenner *et al.* (Zrenner *et al.*, 1995).

Isolation and characterization of LDs

LDs were isolated from Arabidopsis seeds at various stages of development (5, 11 and 17 days after flowering) according to Hanano *et al.* (Hanano *et al.*, 2006). The amount of fatty acids in LDs was determined by a colorimetric method using oleic acid as standard (Nixon and Chan, 1997). Protein concentration in the LDs fraction was estimated by the Bradford assay (Bio-Rad) using bovine serum albumin as a standard (Bradford, 1976). Encapsulation of LDs was evaluated by a simple method based on extraction of lipids with hexane (Tzen *et al.*, 1997). Aggregation and coalescence of LDs as a function of pH was performed by suspending the isolated LDs in 100 mM potassium pyrophosphate at pH values of 4, 5, 6, 7 and 8 and immediately analysing them by light. LDs were subjected to microscopic analysis directly after each extraction without making any

suspension as described before (Hanano *et al.*, 2016a). Microscopic imaging was performed at the magnification of 40× under a LEICA MPS60 microscope and using an Olympus FE-4000 camera. The purity of LDs preparation, their native encapsulation and their number per mL were evaluated by a Flow cytometer (BD FACSCALIBUR, Biosciences, USA). LD size distributions (% frequency) were determined using a laser granulometer (Malvern Mastersizer S; Malvern Instruments, England) fitted with a 320 mm lens as described (White *et al.*, 2006). For TCDD analysis of LDs, the 2,3,7,8-TCDD content was quantified in purified seed LDs by GC/MS using an Agilent Technologies 7890 GC System (USA) coupled to an AMD 402 high-resolution mass spectrometer (Germany). Details of the GC/MS analysis and quality control are described in EPA methods 1613B and 1668A. LD-associated proteins were isolated according to Katavic *et al.* (Katavic *et al.*, 2006) then analysed by SDS-PAGE using 12 % polyacrylamide gels stained with Coomassie Blue R-250. For immunoblotting experiments, proteins were electroblotted onto a PVDF membrane (Millipore) in a Semi-Dry Transfer Cell (Bio-Rad). The membrane was blocked overnight at 4 °C in a solution of 3 % (w/v) bovine serum albumin (BSA) in TRIS-buffered saline (TBS) buffer, pH 7.4. WRI1, α CUL3 and β -actin were immunodetected by incubating the membrane with respective polyclonal antibodies (WRI1 and α CUL3 from GeneScript and β -actin from Sigma-Aldrich, USA) that used in a 1:1000 dilution in TBS buffer (pH 7.4) containing 0.3% (v/v) Tween-20, for 12 h at 4 °C. The signal was detected in a Pharos FX molecular imager (Bio-Rad).

Analysis of gene transcripts

The relative transcriptional abundance of target genes in response to TCDD exposure were analysed by reverse-transcription quantitative PCR (RT-qPCR). Two grams of plant material were used to total RNA extraction using an RNeasy kit according to the manufacturer's instructions (Qiagen, Germany). Reverse transcription reaction (RT) was carried out according to Hanano *et al.* (Hanano *et al.*, 2006). Real-time PCR was performed in 48-well plates using a AriaMx Real-time PCR System (Agilent technologies, USA). Briefly, 25 μ L reaction mixtures contained 0.5 μ M of each specific oligonucleotide

primer for the target and the reference genes (Table S1, Additional file 1.), 12.5 μ L of SYBR Green PCR mix (Bio-Rad, USA) and 100 ng cDNA. QPCR conditions were as described before (Zhu *et al.*, 2012). The relative expression of target genes was normalized using two reference genes *SAND* and *TIP41* (Hu *et al.*, 2009). Each point was replicated in triplicate and the average of C_T was taken. Subsequently, the relative quantification RQ of target genes was calculated directly by the software of the qPCR system. The sequences of amplified regions were confirmed by an ABI 310 Genetic Analyzer (Applied Biosystems) using Big Dye Terminator kit (Applied Biosystems).

Stability Assays

Stability assays were carried out according to Chen *et al.*, (Chen *et al.*, 2013), Arabidopsis seedlings were treated with 20 μ M of the proteasome inhibitor MG132 (Sigma-Aldrich) for 6 h before harvesting at each developmental stage. DMSO was used as mock control and as a solvent for all inhibitor experiments.

Statistics

All data were expressed as means \pm standard deviation (SD). Statistical analysis was carried out using STATISTICA software, version10 (StatSoft Inc.). Comparisons between control and treatments were evaluated by ANOVA analysis. Difference from control was considered significant as $P < 0.05$ or very significant as $P < 0.01$.

Results

TCDD exposure of Arabidopsis plants results in wrinkled seeds with reduced levels of lipid and carbohydrate

Viewed under light microscopy, mature seeds (wild-type Col-0) obtained from TCDD-exposed plants were smaller than controls and had a distinctive 'wrinkled' appearance (Fig. 1A). This wrinkled phenotype was more evident when the seeds were examined under scanning electron microscopy (SEM) (Fig. 1B and C). TLC analysis of lipid extracts revealed a significant reduction of the seed-oil content in seeds from TCDD-exposed plants compared to controls (Fig. 2A). Quantification of fatty acids from storage

triacylglycerols confirmed an 80% reduction in oil content from approximately 3.8 μg per seed in non-exposed controls to 2.1 and 1.0 μg for seeds exposed respectively to 10 and 50 ng L^{-1} TCDD (Fig. 2B). Similar reductions were observed in levels of total seed carbohydrate, which was 1.6 to 3.6-fold lower in seeds of TCDD-exposed plants compared to controls. In contrast, seeds of TCDD-exposed plants contained 1.1 to 1.6-fold higher of proteins compared with controls (Fig. 2B).

Comparison of the fatty acid compositions of triacylglycerols extracted from mature control or TCDD-exposed seeds showed that i) the percentage of 16:0 (palmitic acid) and 18:0 (stearic acid) did not change as a function of TCDD-exposure. ii) percentages of 18:1 Δ^9 (oleic acid) and 18:2 $\Delta^9, 12$ (linoleic acid) were strongly reduced. iii) percentages of 18:3 $\Delta^9, 12, 15$ (α -linolenic acid), 20:0 (arachidic acid), 20:1 Δ^9 (gadoleic acid), 22:0 (behenic acid) and particularly 22:1 Δ^9 (erucic acid) were greatly elevated in the reduced amount of seed oil in TCDD-exposed plants (Fig. 2C). These data showed that exposure of Arabidopsis plants to TCDD results in small, wrinkled seeds with very different lipid and carbohydrate contents compared to controls.

TCDD treatment is associated with increases in LEC1 and WRI1 and decreases in ABI3 and FUS3 transcripts

The much reduced oil content in seeds from TCDD-exposed plants raises the question of whether this decrease is due to a global regulatory effect mediated by LEAFY COTYLEDON1 (LEC1), (LEC2), ABSCISIC ACID INSENSITIVE3 (ABI3), FUSCA3 (FUS3) and WRINLED1 (WRI1), four master transcription factors known as key regulators of fatty acid and TAG biosynthesis/accumulation. In Fig. 3, transcriptome data for *LEC1*, *LEC2*, *ABI3*, *FUS3* and *WRI1* genes show that expression of *LEC1*, *LEC2* and *WRI1* genes was progressively raised after flowering in control plants and that their transcript levels were elevated in TCDD-exposed plants. These increases were about 42, 48 and 33-fold for *LEC1*, *LEC2* and *WRI1*, respectively, by 7 days after flowering (DAF). Inversely, transcript levels of *ABI3* and *FUS3* were greatly reduced in TCDD-exposed plants: the largest decrease was about 17 to 28-fold at 7 to 9 DAF for the two genes respectively. In

contrast, the expression of *ABI3* and *FUS3* genes was higher in control plants at the same time points. These results indicate that TCDD-exposed plants accumulate more of the *LEC1*, *LEC2* and *WRI1* transcripts but less of the *ABI3* and *FUS3* transcripts compared with control plants.

Expression of fatty acid biosynthesis genes was altered in TCDD-exposed plants

The increased expression of transcription factors *LEC1*, *LEC2* and *WRI1* in TCDD-exposed plants raised the issue of whether the expression of downstream target genes was also affected, notably genes involved in the fatty acid biosynthetic pathway. RT-qPCR-analysis of genes transcripts for enzymes of fatty acid biosynthesis was performed as shown in Fig. 4A. The results showed that transcripts of *Accase*, *BCCP2*, *PKP-β1*, *ACP1* and *MCAT* (encoding the first three fatty acid biosynthetic enzymes acetyl-CoA carboxylase, biotin carboxyl carrier protein 2, pyruvate kinase beta subunit 1, acyl carrier protein and malonyl-CoA: ACP malonyltransferase, respectively) were mainly increased between 11 to 15 DAF in control plants but significantly decreased in TCDD-exposed plants. Similarly, transcripts levels of *KASI*, *KASII* and *KASIII* (ketoacyl-ACP synthase I, II and III), which were briefly increased in control plants, actually decreased about 8 to 12-fold in TCDD-exposed plants. Likewise, the expression of *FATA* and *FATB* (acyl-ACP thioesterase A and B, respectively) was highly induced during normal seed development stage, reaching a maximum at 13 DAF. In contrast, transcript levels of both genes were reduced by between 18 and 22-fold in TCDD-exposed plants. Intriguingly, while the transcripts of *FAD2* and *FAD3* (fatty acid desaturase 2 and fatty acid desaturase 3) increased in control plants, these transcripts were even more elevated in TCDD-exposed plants reaching peaks of 29- and 36-fold, respectively by 15 DAF. Finally, transcripts of *DGAT* (diacylglycerol acyltransferase) were more elevated in TCDD-exposed plants than in control plants. The maximal accumulation of *DGAT* transcripts (about 34-fold) was detected in plants treated with 50 ng L⁻¹ TCDD at 15 DAF.

The transcriptome results for fatty acid biosynthesis genes were complemented by metabolome analysis of the major fatty acids during seed development. Firstly, although

the total amount of seed oil increased during seed development, the final amount of seed oil was reduced compared to controls by about 1.5 to 2.4-fold in plants exposed to 10 or 50 ng L⁻¹ of TCDD, respectively (Fig. 4B). Furthermore, when the composition of fatty acids extracted from seeds of non-exposed or TCDD-exposed plants was analysed, percentages of C18:1 and C18:2 were drastically reduced, while those of C18:3, C20:0, C20:1, C22:0 and particularly C22:1 were significantly increased, and the amounts of C16:0 and C18:0 were unaltered (Fig. 4C). Altogether, these results show that TCDD exposure reduces overall fatty acid biosynthesis and selectively alters the net activity of some enzymes thereby changing the final acyl composition of the seed oil.

TCDD exposure affects seed carbohydrate metabolism

As shown in Figure 5A, transcripts of *HXK1* and *G6PDH* genes (encoding hexokinase 1 and glucose-6-phosphate dehydrogenase respectively) were approximately 12-fold more abundant in controls compared with TCDD-exposed plants on day 9 after flowering. On the other hand, the small subunit of ADP-glucose pyrophosphorylase (*ADPase-AP51*) and starch synthase I (*SSI*) encoding genes, *AGPase-AP51* and *SSI*, were increasingly expressed in control plants but significantly decreased in seeds of TCDD-exposed plants. These results were complemented by quantitative analysis of soluble sugars and starch, which showed a 3-fold decrease in their respective amounts in TCDD-exposed plants compared with controls (Fig. 5C and D). These data show that the exposure of plants to TCDD significantly affects carbohydrate metabolism in developing seeds.

LDs-associated proteins were increased by TCDD-exposure

In contrast to lipid and carbohydrate levels, the amount of seed storage proteins increased after exposure to TCDD. Among the major classes of seed proteins are those associated with lipid droplets (LDs). In *Arabidopsis* the major LD proteins are encoded by 11 genes, namely 5 *oleosins* and 6 *caleosins*. As Figure 6A shows, the expression of three oleosin genes, namely *OLEO1*, *OLEO3* and *OLEO5*, was induced by TCDD with maximal transcript levels detected at 7 - 9 DAF. The transcripts of caleosins *CLO1*, *CLO3* and *CLO4*

were also 35 to 40-fold higher in seeds collected from the TCDD-exposed plants than in controls. At biochemical level, LDs were fractioned at the indicated stages of seeds development and their respective protein contents determined. As indicated in Fig. 6B, the total amount of LD-associated proteins was doubled in the samples prepared from TCDD-exposed plants compared with control samples. In parallel, the total enzymatic activity of peroxygenase, an activity found in caleosin proteins, was elevated three fold in LDs of TCDD-exposed plants compared with controls (Fig. 6C). These data suggest that TCDD-exposure induces accumulation of some members of LD-associated proteins and especially the caleosins.

LD morphology was affected in developing seeds exposed to TCDD

The significant alteration in the biochemical composition of LDs raises the question of possible effects on their morphogenesis. To investigate this, LDs were isolated from seeds at 5, 11 and 17 DAF and subjected to morphological characterization. The isolated and purified LD fractions represented about 6.6, 12.8 and 17.9 % of the total control seed weight on 5, 11 and 17 DAF. In contrast, LDs only accounted for 4.1, 5.4 and 6.5 % total weight of seeds exposed to TCDD at the indicated stages. Extraction with hexane, a classical method to determine the native structures of isolated LDs (Tzen *et al.*, 1997) showed that about 86 % of LDs in all preparations had an intact membrane. Light images showed the presence of spherical LDs with a clear surrounding membrane (Fig. 7A). Moreover, the purity and the native structure of LDs were confirmed by flow cytometry. LD size analysis at various stages for control and TCDD-exposed samples showed that the diameters of LDs isolated from control seeds were normally about 2 to 2.2 μm at pH 7.5 (Fig. 7B), while the LDs isolated from TCDD-exposed seeds were twofold smaller at the equivalent pH at each stage (Fig. 7C).

The exposed domains of LD-associated proteins on the surface of the phospholipid monolayer may serve as a physical barrier to prevent aggregation and/or coalescence of LDs at physiological pH (11, 12, 18). However, it is known that isolated LDs aggregate and/or coalesce when the pH of the medium is reduced lower than the isoelectric point

of such proteins (Tzen *et al.*, 1997). To investigate whether isolated LDs from TCDD-exposed seeds might possess different physico-chemical properties, we compared their pH responses with those isolated from control seeds. LDs from control seeds started to aggregate at pH 6 and massively coalesced at pH 5 and 4, whilst the LDs of TCDD-exposed seeds showed a remarkable stability at acidic pH with only few of them coalescing at pH 4 (data not shown). When the diameter of LDs was measured for both samples along the indicated pH range, we found that control LDs were normally about of 2.1 to 2.4 μm at pH 8 to 6 and increased about 4-fold at pH 5 and 4 (Fig. 7D). In contrast, the LDs of TCDD-exposed seeds were slightly larger at pH 4 (2.3 μm in diameter) than they were at pH 7. TCDD-sequestration by LDs was examined using HR/GC-MS as shown in Fig. 7E, which clearly show the presence of a single peak that corresponds exactly to the TCDD standard with a retention time 5.33 min) in organic extracts from the LDs fractioned from seeds of TCDD-exposed plants compared with the extract from LDs of control plants. These results suggest that the TCDD-exposed plant produces LDs that are reduced in size but are more numerous and that possess an increased stability towards pH changes and an enhanced ability to sequester TCDD.

WRI1 is degraded by 26S proteasome in TCDD-treated plants

The TCDD-transcriptional activation of *WRI1* (Fig. 3) did not lead to significant activation of fatty acid biosynthesis genes (Fig. 4A). This led us to investigate whether TCDD-exposure might instead be associated with the post-transcriptional or post-translational regulation of *WRI1*. The ubiquitination/degradation of *WRI1* protein via the 26S proteasome and possibly a CRL3BPM E3 ligase is suggested to be one of the most potent mechanisms involved in the regulation of *WRI1* biosynthesis (Chen *et al.*, 2013). When we checked this possibility, we found that the *WRI1* protein was indeed highly degraded in TCDD-exposed plants compared with controls (Fig. 8A). In parallel, transcript levels of a 14-subunit core protease (CP) of 20S proteasome were differentially increased as a function of TCDD-exposure in developing seeds coincidentally with *WRI1* disappearance. In particular, the transcripts levels of the subunits *PAA1*, *PAC1*, *PAF1*, *PBA1*, *PBB1*, *PBD1*

and *PBE1* were greatly increased in TCDD-exposed plants at 11 DAF (Fig. 8B). Furthermore, Inhibition of proteasome by MG132, a cell permeable proteasomal inhibitor, restored the accumulation of WRI1 protein in TCDD-exposed developing seeds but did not affect stability of WRI1 protein in non-exposed plants (Fig. 8C). Additionally, the interaction of WRI1 with α CUL3-based ligase was demonstrated by immunoprecipitation using WRI1 or α CUL3 antibodies. The WRI1 antibody successfully precipitated α CUL3 from protein plant extracts in the controls much more effectively than in TCDD-exposed plants. Inversely, α CUL3 antibody successfully precipitated WRI1 (Fig. 8D). These data suggest that the instability of the WRI1 protein in TCDD-exposed plants might be regulated by a 20S proteasome-dependent process.

Discussion

TCDD, which is the most toxic congener of the dioxin group of xenobiotics, is a highly persistent organic pollutant that can severely impact on animal and human health (McConkey *et al.*, 1988; Carney *et al.*, 2006). Here we have now shown that TCDD can have very specific effects on aspects of plant development, and especially on seed maturation in Arabidopsis. These effects appear to be mediated via interactions of TCDD with some of the major transcription factors involved in mediating the flux of assimilates towards storage compounds in seeds. TCDD-treated Arabidopsis plants yielded highly abnormal seeds that were smaller than controls, with a wrinkled phenotype and severe reductions in levels of lipid and carbohydrate. Such a phenotype bears a close resemblance to the *WRI1*-deficient mutant phenotype, as reported in Arabidopsis (*wrinkled1*) and other plants, and which is also characterized by low-seed-oil and abnormal carbohydrate metabolism (Focks and Benning, 1998).

This suggests a possible involvement of key regulators of storage compound biosynthesis in the TCDD-induced phenotype. In line with these observations, decreases in the size of seeds and their oil content were previously reported in Arabidopsis plants exposed to TCDD (Hanano *et al.*, 2015b). Moreover, a negative regulatory effect of

TCDD on fatty acid, cholesterol and carbohydrate metabolism was also demonstrated in mouse liver (Lakshman *et al.*, 1988;Angrish *et al.*, 2013). The deficiency in seed lipid and carbohydrate levels could be due to a direct reduction in their respective anabolic pathways and/or to an activation of their catabolic pathways. The increase in seed protein levels in such seeds is not surprising since an inverse relationship between oil and protein accumulation in seeds has been reported for a range of plant species including *Arabidopsis* (Siloto *et al.*, 2006;Eskandari *et al.*, 2013). In addition, TCDD treatments led to the induction of two relatively abundant proteins, lipoxygenase and caleosin/peroxygenases that metabolize fatty acids from storage and membrane lipids to oxylipins, hence further increasing protein and decreasing lipid in the seeds (Hanano *et al.*, 2016c).

In order to gain further insights into the molecular regulation of oil/carbohydrate metabolism during seed maturation of *Arabidopsis*, gene expression patterns of the master transcription factors *LEC1*, *LCE2*, *WRI1*, *ABI3* and *FUS3* that regulate storage compound accumulation in *Arabidopsis* (Focks and Benning, 1998;Mu *et al.*, 2008b) were investigated. In seeds from TCDD-exposed plants, the dearth in seed reserves was consistent with reduced levels of *ABI3* and *FUS3* expression compared to controls. For example, *Arabidopsis* plants with mutations in *abi3* and *fus3* genes exhibit similar defects in the accumulation of seed reserves, acquisition of desiccation tolerance, reduction of chlorophyll, and inhibition of anthocyanin accumulation (Keith *et al.*, 1994a;Parcy *et al.*, 1997). Moreover, a particular abscisic acid (ABA)-insensitivity is observed in *abi3* mutant but not in *fus3* mutants suggesting collaborative and independent roles of *ABI3* and *FUS3* on various ABA-related processes during *Arabidopsis* seed development. In particular, *FUS3*, which was characterized initially as a transcription factor with a B3-domain, binds the RY cis-motif in promoter regions of many seed specific genes. Greatly reduced numbers of LDs were found in cotyledons of developing seeds of *fus3* mutants (Keith *et al.*, 1994b), and the total fatty acid content in *fus3* seeds was one-third of WT seeds . Despite *FUS3* and *ABI3* being synchronously

downregulated by TCDD, *LEC1* and *LEC2* were significantly upregulated. There is no definitive evidence that excludes the possibility that *LEC1* and *LEC2* can directly act on the promoters of fatty acid biosynthetic genes. However, genetic analysis indicates that the *LEC1* function is partially dependent on *ABI3*, *FUS3*, and *WRI1* in the regulation of fatty acid biosynthesis (Mu *et al.*, 2008b). *FUS3* acts exclusively as a transcriptional activator and the regulation of microRNA-encoding genes is one mechanism by which *FUS3* may indirectly repress target genes. *FUS3* also directly up-regulates VP1/*ABI3*-LIKE1 (*VAL1*), which encodes a repressor of transcription involved in the transition from embryo to seedling development (Wang and Perry, 2013). In this context, the down-regulation of *ABI3* and *FUS3* can be a possible explanation as to why TCDD-exposed Arabidopsis plants had reduced chlorophyll, lower seed yields and much reduced rates of seed germination.

Unexpectedly, our results showed that seeds from TCDD-exposed plants, with a wrinkled phenotype and much-reduced amounts of seed storage reserves, actually displayed considerably elevated levels *LEC1*, *LEC2* and *WRI1* transcripts. Such increases in transcripts levels are inconsistent with previous findings indicating that; i) overexpression of *LEC1* normally causes globally elevated level of seed lipid accumulation (Mu *et al.*, 2008a) – contrary to what was observed in our TCDD-exposed plants; ii) *LEC1* acts as a positive regulator upstream of *ABI3*, *FUS3* and *WRI1* (Baud and Lepiniec, 2009; To *et al.*, 2012). Our findings that the reduction of *ABI3* and *FUS3* transcripts is associated with serious defects in the seed maturation process consistent with several lines of genetic, molecular and biochemical evidence. In contrast, the transcriptional activation of *LEC1*/*WRI1* conflicts with published data because a such activation did not lead to increases in levels of seed oil (Cernac and Benning, 2004). To investigate this apparent inconsistency, transcriptome and metabolome analysis for the major components of fatty acid and carbohydrate metabolism was performed. Surprisingly, the transcripts levels of genes encoding key enzymes involved in the fatty acid biosynthesis were decreased, while the transcripts of the genes that govern their

desaturation and TAG assembly were increased. These transcriptional changes were associated with a reduction in the overall amount of seed oil and of oleic and linoleic acids plus a relative increase in levels of very long chain fatty acids.

Although there is no comparative data on the effect of persistent xenobiotics such as dioxins on the plant lipidome, similar alternations in lipid content and FAs composition were reported when marine animals exposed to such compounds. It was suggested therefore the lipidome “signature” could be used as a biomarker to assess the severity of effective exposure to dioxins. Furthermore, in the context of plant responses to environmental stress, several lines of biochemical evidence have indicated that extreme environmental factors including high temperature, severe drought, high salinity, high nitrogen, and heavy-metal stress tend to decrease seed oil content and increase the percentage of unsaturated fatty acids (Canvin, 1965; Flagella *et al.*, 2002; Hou *et al.*, 2006). One of the biological implications of the increasing level of polyunsaturated fatty acids (PUFAs) has been demonstrated by a particular modification in the composition of the cell membrane fatty acids (FAs) under the activation of adjacent membrane-bound desaturases adjusting therefore the membrane permeability and insuring an effective acclimation of plants to environment (Williams *et al.*, 1988; Falcone *et al.*, 2004). Other possible roles of PUFAs, in particularly C18:2 and C18:3, is their use as substrates in the biosynthesis of the most active plant oxylipins via the lipoxygenase (LOX) pathways (Feussner and Wasternack, 2002). Interestingly, a coordinated increase in transcript levels of 9-LOX and 13-LOX genes with the accumulation of their corresponding FA-hydroperoxides deriving from linoleic or linolenic acids in Arabidopsis exposed to TCDD was recently reported (Hanano *et al.*, 2015b).

Our results showed that the degradation of WRI1 protein in the TCDD-exposed is coordinated with the up-regulation of some specific proteolytic subunits of 20S proteasome and the inhibition of the proteasome machinery by MG132 blocked the TCDD-induced turnover of WRI1. This result suggests that the regulation of fatty acid biosynthesis in the TCDD-exposed plants may be governed by

ubiquitination/degradation of the WRI1 protein via the 20S proteasome. In line with this, the involvement of 26S proteasome-induced ubiquitination/degradation of WRI1 protein was demonstrated in Arabidopsis plants and this had widespread effects on plant development and fatty acid content in mutant seeds (Chen *et al.*, 2013). Our observations are supported by several lines of molecular, biochemical and genetic evidence suggesting a pivotal role the ubiquitin/26S proteasome machinery in plant developmental process and its tolerance to oxidative stress (Wang *et al.*, 2009), in plant hypersensitive responses (Dahan *et al.*, 2001) and in plant–virus interactions during infection (Sahana *et al.*, 2012).

The involvement of 26S proteasome as part of the response to TCDD exposure has also been demonstrated in animals. For example, it was reported that the 26S proteasome degraded the TCDD-receptor, the Aryl Hydrocarbon Receptor (AHR), when hepa1c1c7 mouse cells were exposed to TCDD. Also, the inhibition of the 26S proteasome by MG132 blocked the TCDD-induced turnover of AhR and subsequently increased the accumulation of *CYP1A1* gene transcripts, encoding a first-line enzyme in the detoxification of such xenobiotics (Ma and Baldwin, 2000). It is interesting therefore that the induced 26S proteasome-degradation of WRI1 resulted in a phenotype which is quite similar to *wrinkled1* phenotype, a mutant of Arabidopsis initially characterized by a deficiency in the seed-specific regulation of carbohydrate metabolism (Focks and Benning, 1998). This is in agreement with our results that seeds from TCDD-exposed plants showed reduced carbohydrate accumulation. Moreover, in *wri1* mutant lines, the lack of transcriptional activation of the fatty acid biosynthetic pathway in early maturing embryos is responsible for a severe defect in TAG biosynthesis that results in the production of wrinkled seeds depleted in oil (Focks and Benning, 1998). Inversely, Arabidopsis lines overexpressed *wri1* were typified by an increasing seed oil content (Cernac and Benning, 2004). More intriguingly, the overexpression of *WRI1* in *Brassica napus* resulted in the up-regulation of genes involved in glycolysis, FA synthesis, lipid assembly, and flowering. Membrane lipid profiling showed increased levels of the

membrane lipids, monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and phosphatidylcholine (PC) in leaves, but reduced level of DGDG, MGDG and increased levels of PC, phosphatidylethanolamide, and oil [triacylglycerol (TAG)] in the siliques during early seed development stage plus a positive effect on flowering and oil accumulation (To *et al.*, 2012; Li *et al.*, 2015). This supports our previous data which showed that TCDD-exposed *Arabidopsis* plants were delayed in flowering (Hanano *et al.*, 2015b) suggesting a key role of *WRI1* in several diverse plant responses to TCDD exposure.

We have shown in date palm seedlings that TCDD-exposed plants had increased levels of some oleosin and caleosin gene transcripts, notably *CLO4*, which was accompanied by increased peroxygenase enzyme activity (Hanano *et al.*, 2016a). Caleosins can act as peroxygenases are involved in oxylipin metabolism, a variety of developmental processes, and a range of biotic and abiotic stress responses in both plants and fungi. In contrast, oleosins confer a remarkable stability that prevents aggregation or coalescence of LDs over a wide range of environmental conditions (Leprince *et al.*, 1998; Beisson *et al.*, 2001). Intriguingly, the formation of small LDs with increasing amounts of LD-associated proteins seems to be a strategy by which plants can sequester certain classes of toxic hydrophobic pollutants. These findings strongly support our data showing that LDs isolated from TCDD-exposed seeds had a reduced size and elevated resistance to aggregate/coalesce at acidic pH. In fungi, the protective effect of LDs was limited to lipophilic toxins and LDs were ineffective against more polar antibiotic agents, such as terbinafine or caspofungin (Chang *et al.*, 2015). Larger fungal LDs were more effective protective agents than smaller LDs as they were able to sequester larger quantities of lipophilic toxins. In date palm the most effective anti-dioxin protective activity was found in LDs with a size range of about 1-2 μm (Hanano *et al.*, 2016a), which is similar to the most abundant size range in the TCDD-exposed seeds of *Arabidopsis*. Together, these data suggest that intracellular LDs may play important roles in the

response of a wide range of organisms, from plants and animals to fungi, to potentially toxic xenobiotic agents such as dioxins.

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List of abbreviations

LDs, lipid droplets: CLO, caleosin: OLEO, oleosin: TCDD, 2,3,7,8-polychlorinateddibenzo-p-dioxins: TAG, triacylglycerol: LOX, lipoxygenase: PXG, peroxygenase: WRI1, wrinkled1: LEC, leafycotyledon: ABI2, abscisic acid insensitive3: FUS3, fusca3: Accase, acetyl-CoA carboxylase: ACP1, acyl carrier protein: MCAT, malonyl-CoA: ACP malonyltransferase: KASI, ketoacyl-ACP synthase: FAD3, fatty acid desaturase: HXK1, hexokinase 1: G6PDH, glucose-6-phosphate dehydrogenase: ADPase-ApS1, ADP-glucose pyrophosphorylase: SSI, starch synthase I:

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Figure legends

FIGURE 1. Wrinkled-like phenotype of mature seeds following TCDD-exposure. (A) Micrographs of mature seeds (wild-type Col-0) of control plants (0) and from plants exposed to 10 and 50 ng L⁻¹ TCDD taken at 30X magnification. **(B)** and **(C)** Scanning Electronic Microscopy (SEM) of the whole seeds from control and TCDD-exposed plants or for a focusing zone on their respective surfaces at magnification of 200× or 500×, respectively. Scans were performed using a Vega II XMU, TESCAN, Czech Republic. Bars represent 200 µm and 50 µm for both set of photos in **(B)** and **(C)**, respectively.

FIGURE 2. Biochemical composition of mature seeds from TCDD-exposed plants. (A) Qualitative TLC of lipid extracts from mature seeds of control or TCDD-exposed plants. Triacylglycerols (TAG) were visualized by exposure to iodine vapour and compared with a standard mixture TAGs (S). **(B)** Relative amounts of TAGs, carbohydrate and proteins in TCDD-exposed seeds compared with controls. **(C)** Fatty acid composition of TCDD-exposed seeds compared with controls. Values are mean ± SD (*n* = 6) of three measurements for each treatment. Asterisks indicate significant differences in fatty acids composition between non-exposed and TCDD-exposed plants (* *P* < 0.05; ** *P* < 0.01).

FIGURE 3. Heat map of the effects of TCDD treatments on the expression levels of selected transcription factors involved in the regulation of seed development. Seeds from control plants or TCDD-exposed plants to 10 and 50 ng.L⁻¹ TCDD were analysed

from 5 to 17 days after flowering at 2-day intervals. RNAs were isolated and their respective cDNAs were prepared. Gene transcripts were analysed by qRT-PCR as described in Methods. Three independent measurements were taken of cDNAs prepared from three individual plants for each treatment. The colour scale (red-white-green) indicates relative changes of transcript abundance of -50, 1 and +50 fold, respectively. For each stage, the expression level for a given gene in seeds unexposed to TCDD was defined as 1, and the corresponding abundance changes under 10 and 50 ng.L⁻¹ TCDD were calculated directly using the Applied Biosystems qPCR system software.

FIGURE 4. Effects of TCDD on transcriptional and post-transcriptional components of the fatty acid biosynthesis pathway in the seeds of Arabidopsis. (A) Transcriptional analysis of key genes involved in the synthesis, elongation, desaturation and assembly of fatty acids as determined by qRT-PCR. Data are mean values \pm SD ($n = 6$) of three measurements of cDNAs prepared from three individual plants for each developmental stage. The colour scale (white-green-black) indicates relative changes in transcript abundance of 1, 25 and 50 fold, respectively. For each stage, the expression level for a given gene in seeds unexposed to TCDD was defined as 1. (B) Seed oil content expressed as % seed dry weight. (C) Fatty acid composition in seeds after TCDD-exposure compared with controls. Data are mean values \pm SD ($n = 6$) of three measurements were taken in three individual plants for each treatment. Different lowercase letters indicate significant differences ($P < 0.05$) in oil content between various developmental stages. Asterisks indicate significant differences in oil content or fatty acids composition between non-exposed and TCDD-exposed plants (* $P < 0.05$; ** $P < 0.01$).

FIGURE 5. TCDD affects carbohydrate metabolism in Arabidopsis seeds. (A) Transcript levels of selected carbohydrate metabolism genes were analysed by qRT-PCR. Data are mean values \pm SD ($n = 6$) of three measurements of cDNAs prepared from three individual plants for each developmental stage. The colour scale (red-white-green) indicates relative changes of transcript abundance of -50, 1 and +50 fold, respectively.

For each stage, the expression level for a given gene in seeds unexposed to TCDD was defined as 1 and the relative abundance changes under 10 and 50 ng.L⁻¹ TCDD were calculated. **(B)** and **(C)** Levels of soluble sugars and starch during seed development in TCDD-exposed plants versus control plants. Values are mean \pm SD ($n = 6$) of three measurements in three individual plants for each dose of TCDD.

FIGURE 6. Regulation of oleosin and caleosin encoding genes expression as a function of TCDD treatment. **(A)** Transcriptional analysis of OLEO and CLO-encoding genes was performed at various stages of the seed development in the presence or absence of TCDD. Data are mean values \pm SD ($n = 6$) of three measurements taken in three cDNAs prepared from three individual plants for each developmental stage. The colour scale (white-green-black) indicates relative changes of transcript abundance of 1, 25 and 50 fold, respectively. **(B)** Immunodetection of caleosins in LDs fractioned from seeds at various developmental stages using a polyclonal antibody prepared from the complete sequence of Clo1 from *A. thaliana* used at dilution of 1:500 in TBS buffer (pH 7.4). The secondary antibody was horseradish peroxidase-conjugated anti mouse IgG (Sigma-Aldrich, USA), diluted 1:2000. The signal was detected in a Pharos FX molecular imager (Bio-Rad). **(C)** Caleosin/Peroxygenase enzymatic activities associated with LDs during seed development as a function of TCDD treatment. Hydroxylation of aniline was measured in LDs prepared from seeds at various stages after administration of TCDD at 0, 10 and 50 ng L⁻¹. Three independent experiments were analysed and data averaged.

FIGURE 7. Effects of TCDD-exposure on the physico-chemical properties of LDs isolated from Arabidopsis seeds. **(A)** Light micrographs of the isolated LDs from seeds of Arabidopsis treated or untreated with TCDD on day 17 after flowering. LDs suspended in 100 mM potassium pyrophosphate at pH 7.4 were observed under a LEICA MPS60 microscope and the images viewed at a magnification of 40 \times . Bar represents 5 μ m. **(B)** and **(C)** Size distributions (% frequency) of LDs isolated from non-exposed or TCDD-exposed seeds, respectively, at different developmental stages. **(D)** Evaluation of LD diameters (μ m) after their aggregation and/or coalescence as a function of pH medium.

LDs sizes were determined using a laser granulometer as described in Methods. **(E)** GC/MS analysis showing a pronounced TCDD peak (Retention time \approx 5.33) in LDs isolated from seeds on day 17 compared with the respective control. Three measurements were done for three individual plants. Data are mean values \pm SD ($n = 6$).

FIGURE 8. Degradation of WRI1 protein in TCDD-exposed Arabidopsis seeds. **(A)** Western blot analysis of WRI1 protein levels in control and TCDD-exposed seeds at various stages of development. β -actin is used as a loading control in western blot analysis. **(B)** Transcriptional analysis of the genes encoding fourteen subunits of proteasome 20S at various stages of seed development in the presence or absence of TCDD. Data are mean values \pm SD ($n = 6$) of three measurements of cDNAs prepared from three individual plants for each developmental stage. The colour scale (white-green-black) indicates relative changes of transcript abundance of 1, 25 and 50 fold, respectively. **(C)** Stabilizing essay of WRI1 protein by inhibition of proteasome activity with MG123. **(D)** Immunoprecipitation (IP) of the complex WRI1- α CUL3 with WRI1 or with α CUL3 antibodies. Data show co-precipitation of α CUL3 with WRI1 in TCDD-exposed protein extracts at days 5, 11 and 17 post-flowering compared with controls. About 40 mg of total protein extract was loaded and immunodetection performed using α CUL3 or WRI1 antibodies, respectively. Note that the α CUL3 and WRI1 immunoblots have been cropped to maximize clarity as there were no non-specific bands present on the membranes.

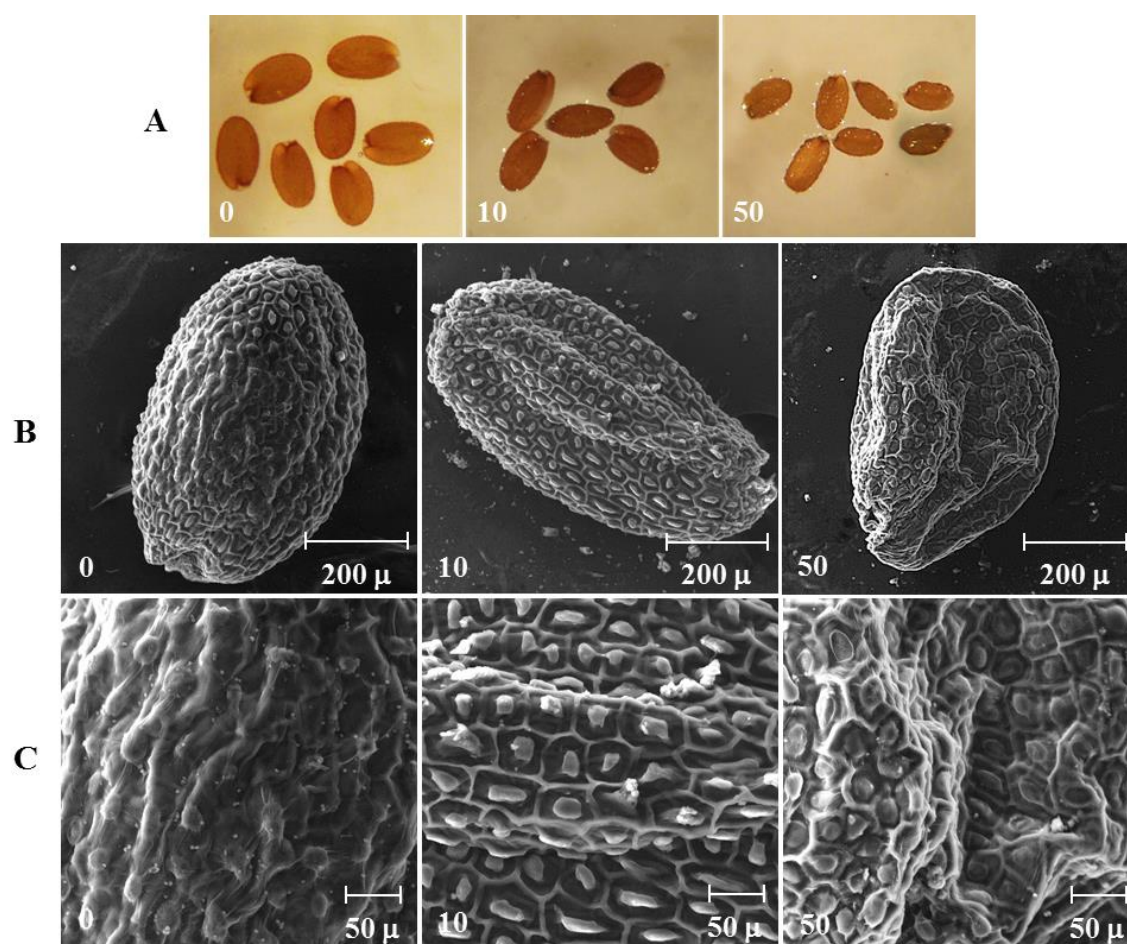


Figure 1.

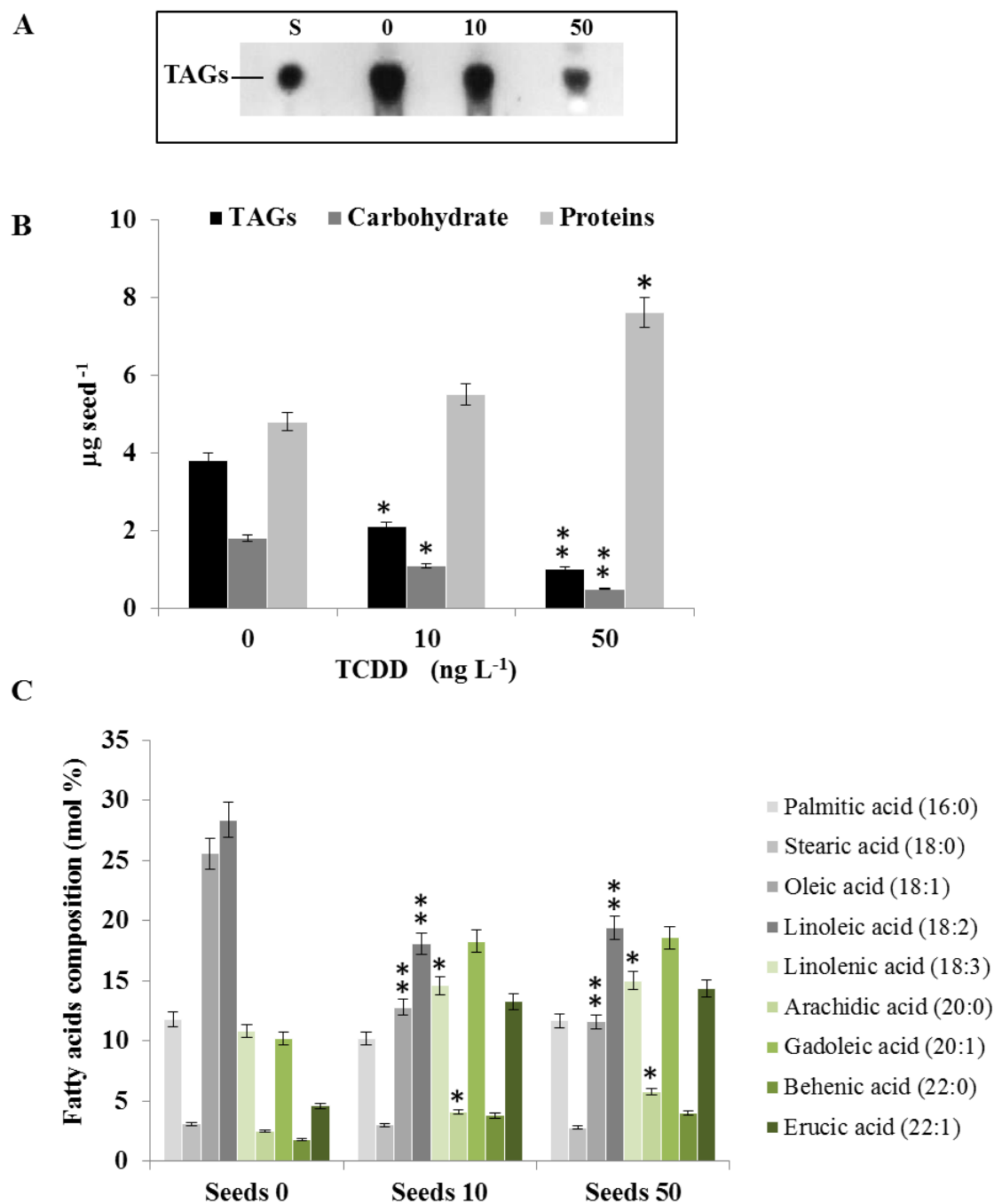


Figure 2.

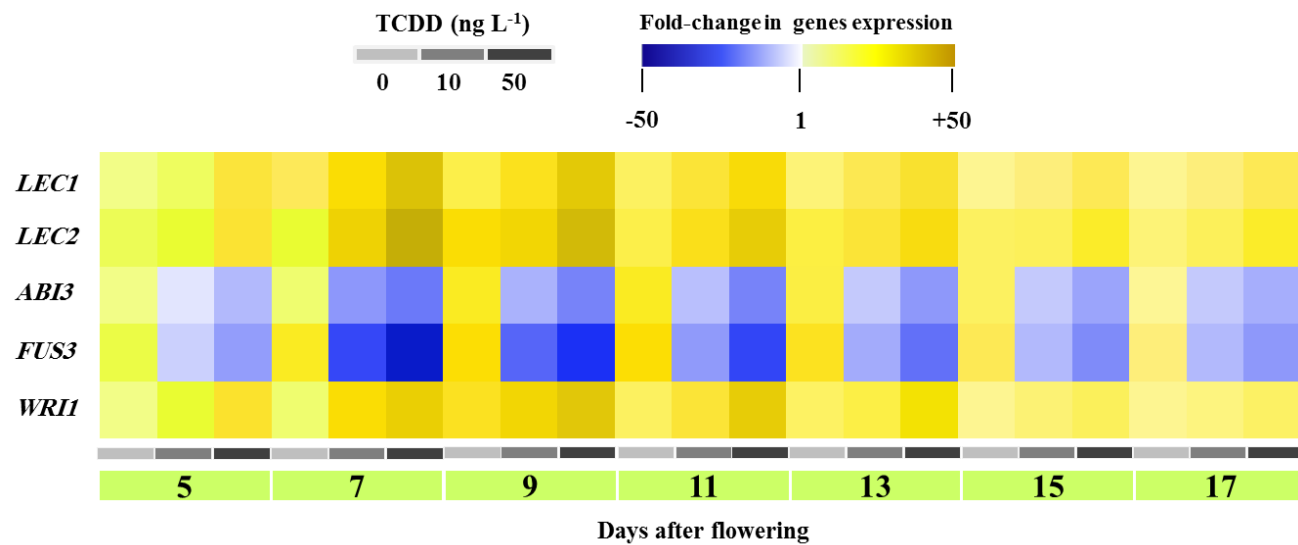


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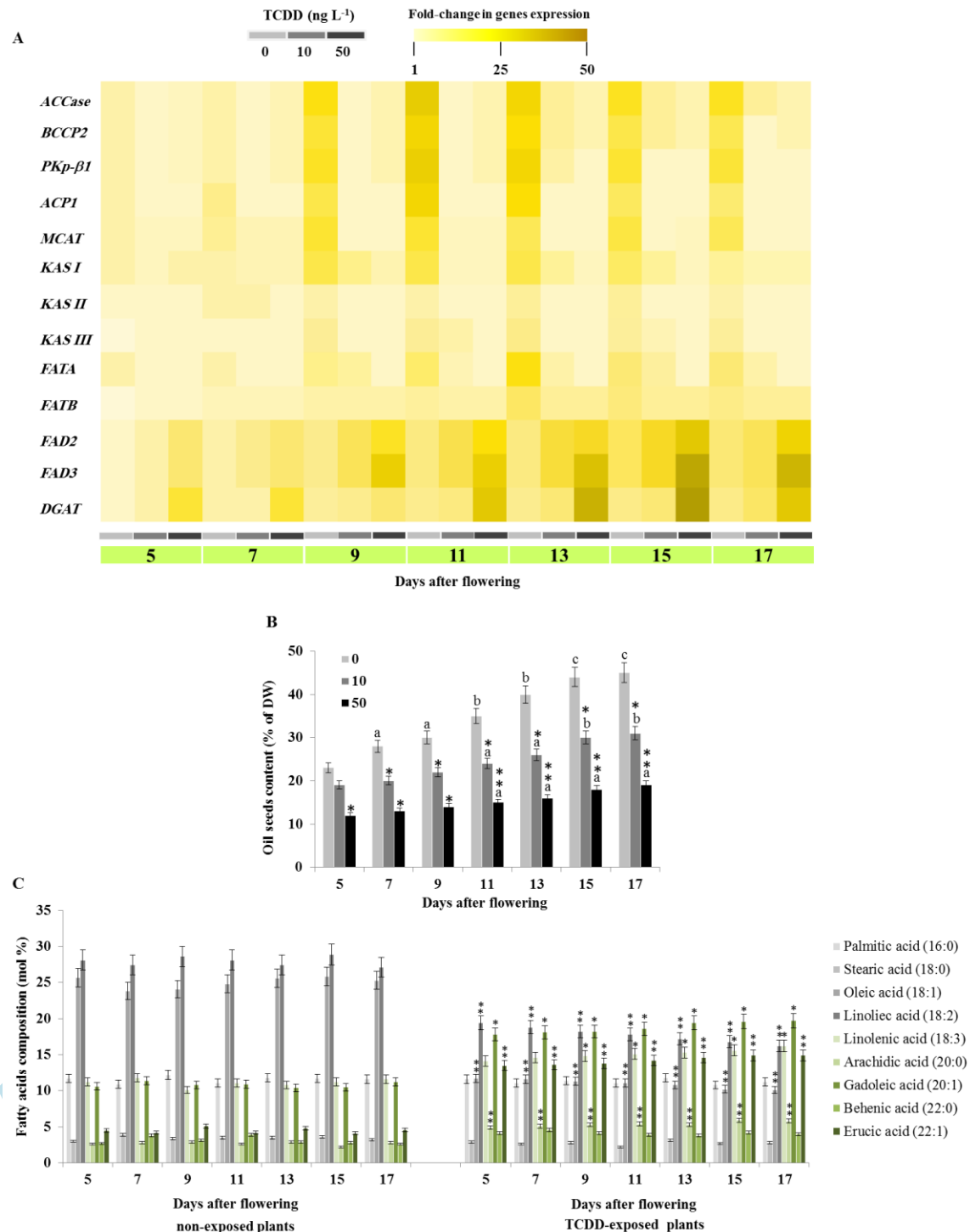


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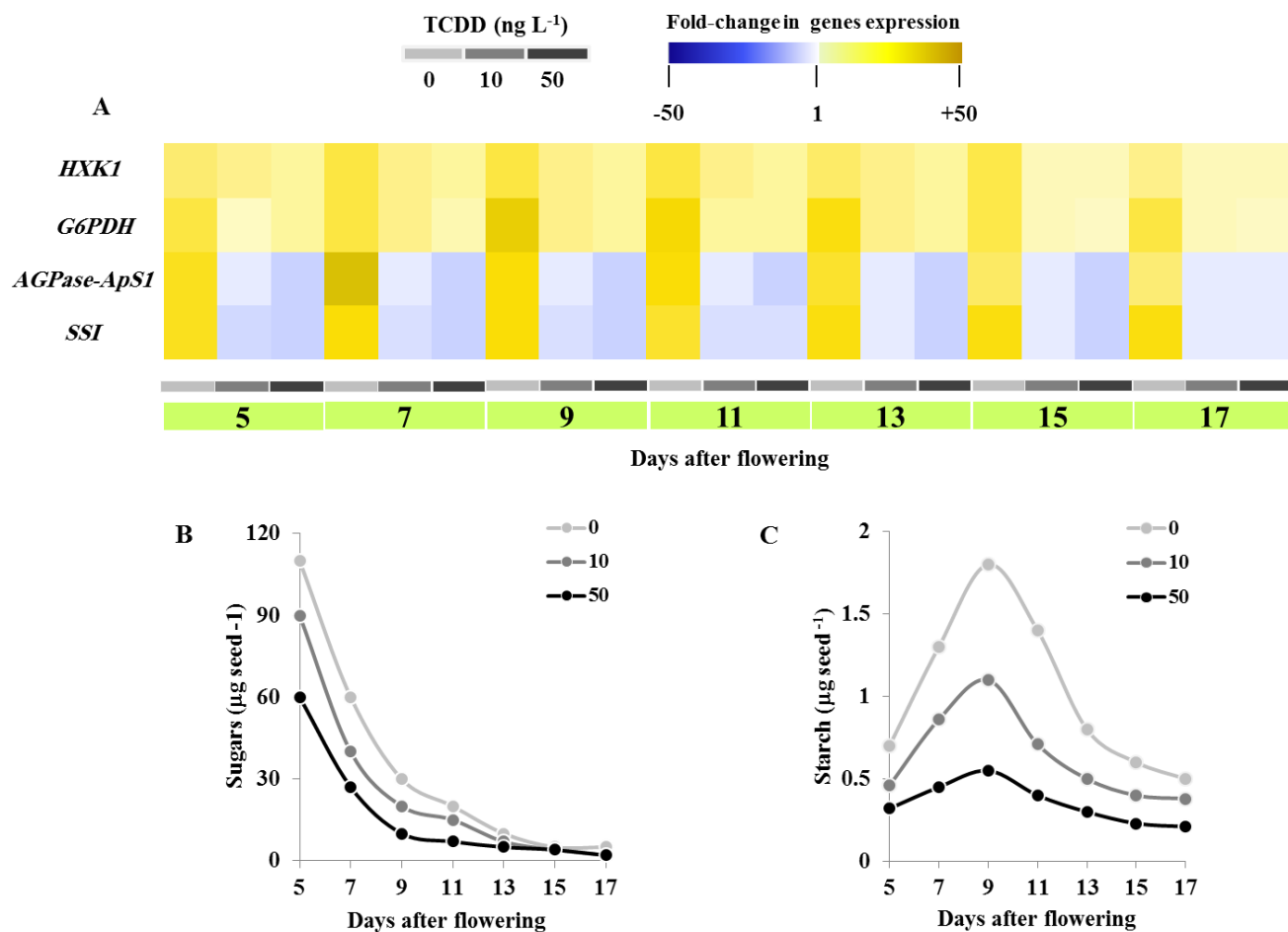


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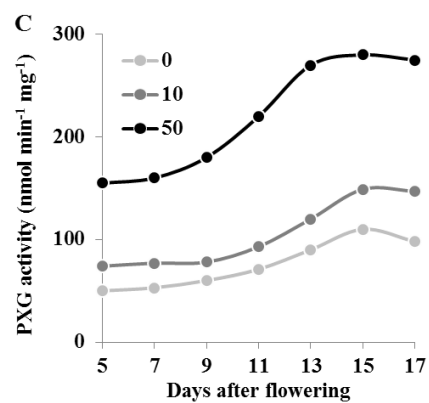
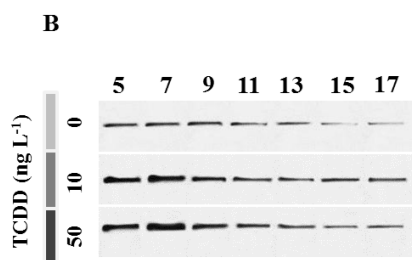
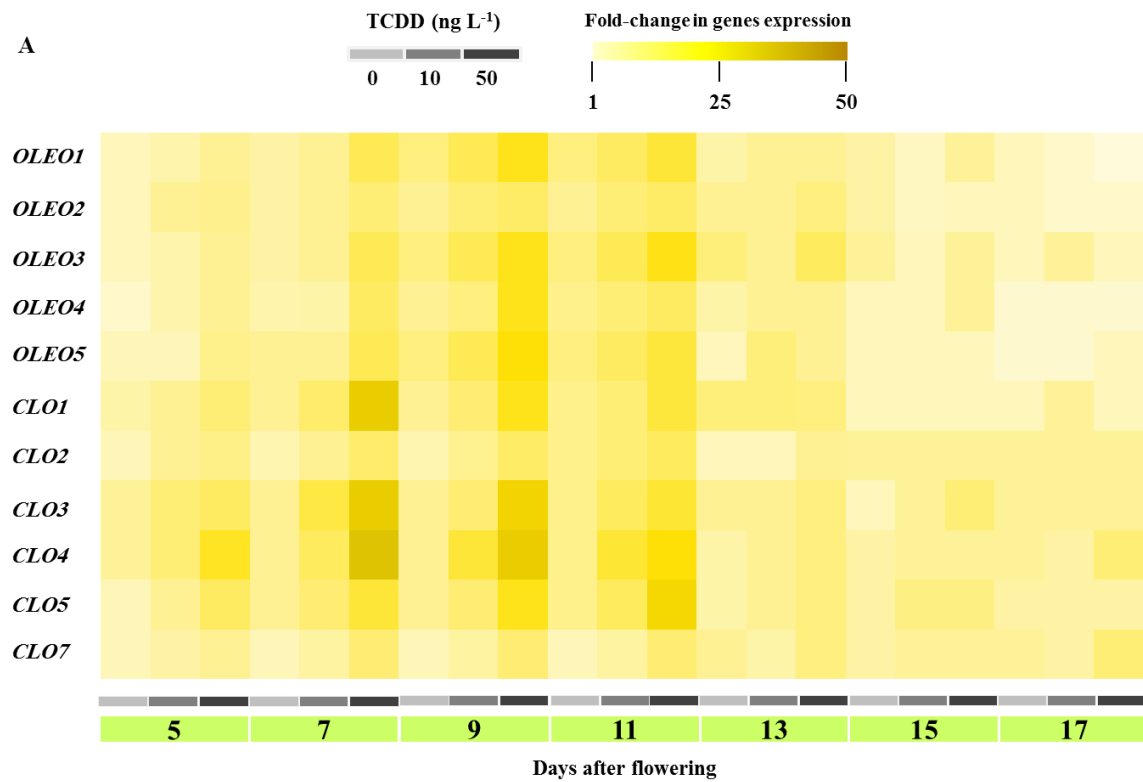


Figure 6.

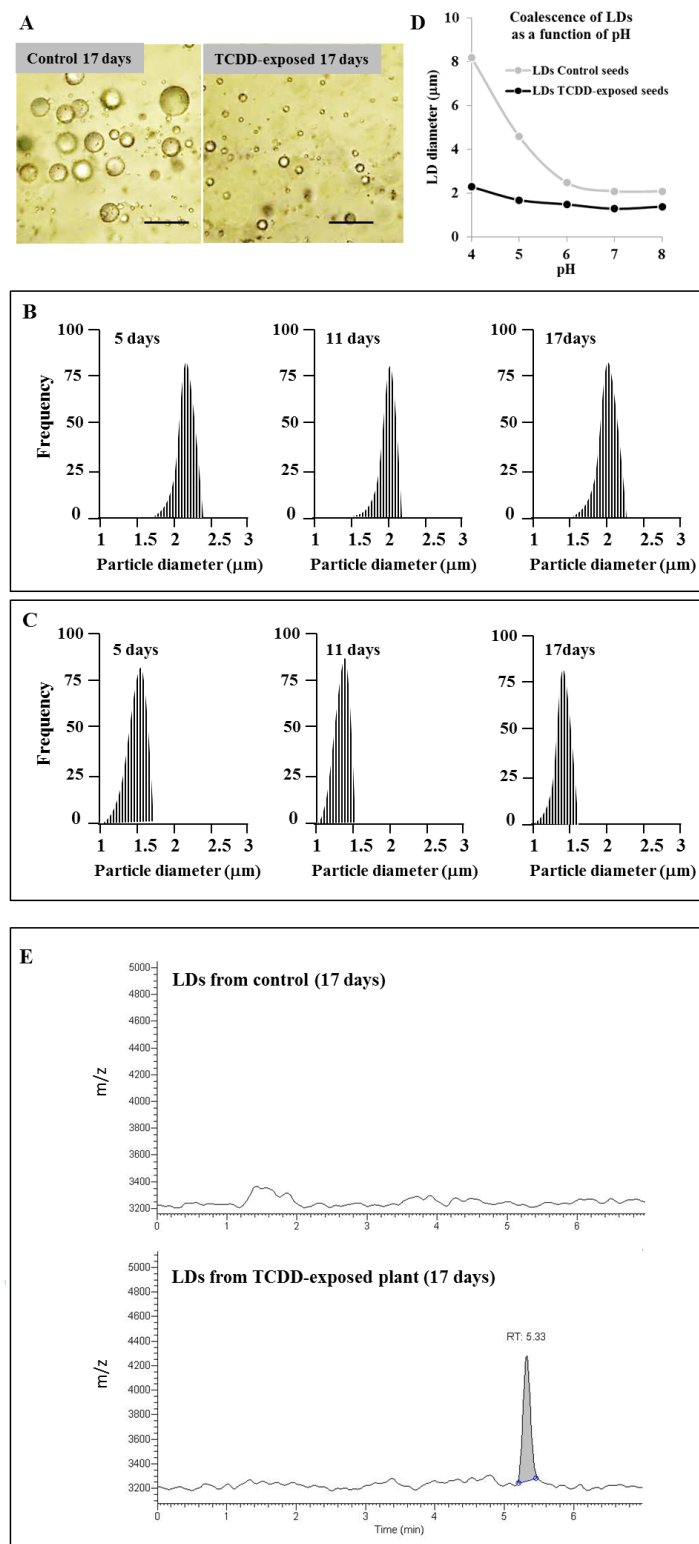


Figure 7.

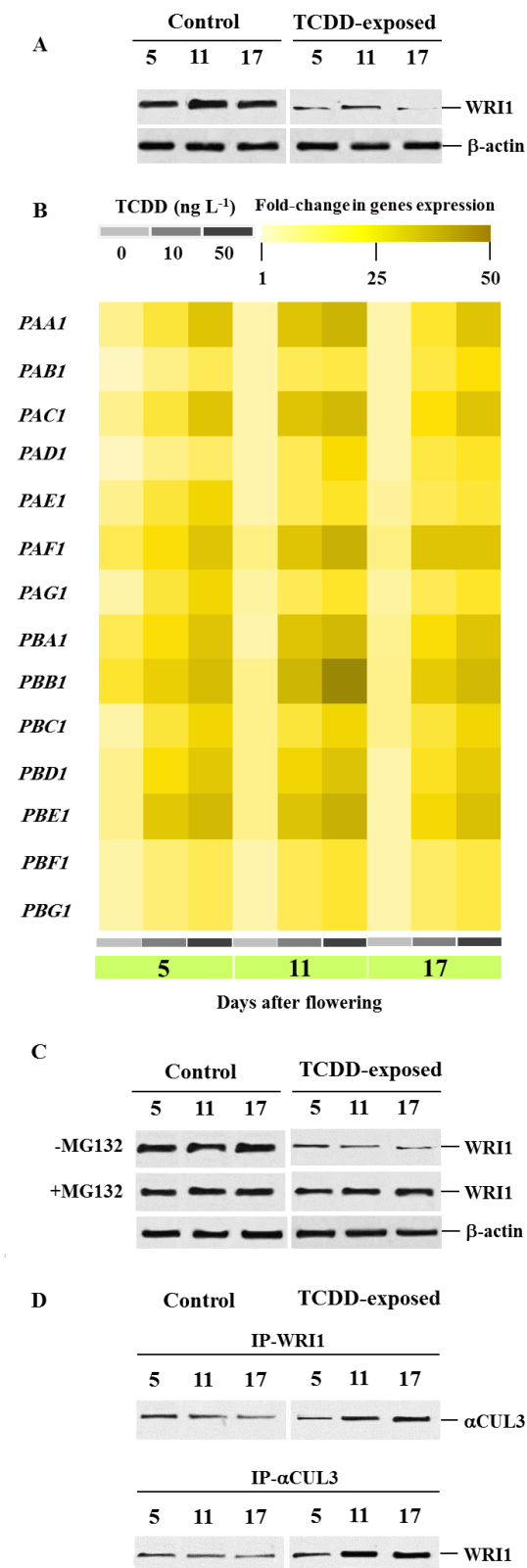


Figure 8.